REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 96-99 and 103-114 are pending in this case. Claims 72-95 have been withdrawn under a restriction requirement for a non-elected invention. Claim 114 has been rejected under 35 U.S.C. § 112 and Claims 96-99 and 103-114 have been rejected under 35 U.S.C. § 103. Claims 96, 97 and 108 have now been amended. Claims 103-106, 109 and 114 have now been cancelled. New claim 115 has now been added.

35 U.S.C. § 112, Second paragraph Rejection

The Examiner has rejected Claim 114 as being indefinite as it does not define the term EGF. Please note that Claim 114 has now been cancelled. Claim 96, which now contains the limitation of EGF has been amended so as to include the full name of EGF, thereby overcoming the Examiner's rejection.

35 U.S.C. § 103(a) Rejection

Claims 96-99 and 103-114 have been rejected under 35 U.S.C. 103(a) as being obvious over WO 94/23050 in view of Yamazaki et al (Journal of the National Cancer Institute, 1998, Vol. 90;581-587), Farrell et al and Ogris et al (Journal of Biological Chemistry, 2001, Vol 276: 47550-47555).

The Examiner states that WO94/23050 teaches a method of blocking RNA translation by administering a gene encoding an RNA which hybridizes and inhibits the function of a cellular RNA, the RNA being complexed with a carrier comprising a cell-specific binding agent and a gene-binding agent. The Examiner further states that WO94/23050 teaches the cell-specific binding agent is specific for a cell surface structure and the gene-binding agent is a compound such as a polycation. The Examiner further states WO94/23050 teaches that the antisense RNA is a ribozyme.

The Examiner states that Yamazaki et al teach a method of killing cells by administering a ribozyme in a nucleic acid carrier. The Examiner states that the ribozyme comprises a targeting moiety by virtue of containing regions of complementary sequence. In addition, the Examiner states that the dsRNA ribozyme downregulates expression of an aberrant EGFR receptor.

The Examiner also cites Farrell et al which teaches that double stranded RNA mediates apoptosis via up-regulation of IFN-α/ß.

In addition, the Examiner notes that Ogris et al teach that melittin, PEI, DNA complexes exhibit higher transfection efficiency than PEI/DNA complexes alone.

The Examiner then goes on to suggest that it would be obvious to devise a method of cell killing based on the combined teachings of WO94/23050, Farrell et al and Yamazaki and further it would be obvious to include melittin based on the teachings of Ogris.

The Examiner's rejection is traversed.

Applicant disagrees with the Examiner when she writes that the term "targeting moiety" has not been defined (see page 10, 4th paragraph of the office action of 27 May 2009). In response to the last office action 26 November 2008, Claim 96 was amended to define the targeting moiety to "a ligand or antibody which binds to a cell surface marker being specific to the target cell and/or tissue". The "targeting moiety" of Yamazaki is an RNAzyme which hybridizes to an RNA encoding a cell surface receptor – the EGF receptor. Introduction of this ribozyme into a tumor cell expressing this receptor leads to cleavage of the RNA molecule encoding the receptor. This ultimately decreases the ability of such cells to generate tumors and inhibits their growth. **It does not lead to the death of the tumor cells**.

Thus, contrary to what the Examiner maintains in the office action (see page 10, 4th paragraph of the office action of 27 May 2009), Yamazkai does not teach cell killing. Since neither WO94/23050 nor Yamazaki teach agents that are capable of cell killing, Applicant maintains that the double stranded RNA of the presently claimed invention is not functionally equivalent to the ribozyme of Yamazaki nor the dsDNA ofWO94/23050, nor are they used for the same purpose, as indicated by the Examiner on Page13, first paragraph of the office action of 27 May, 2009. Accordingly, Applicant maintains that one would not have been motivated to substitute the double stranded RNA molecule of Yamazaki or the double stranded DNA molecule of WO94/23050 with a double stranded RNA molecule which induces viral-like double stranded mediated apoptosis.

In order to expedite prosecution of this case, the claims have now been amended to limit the compositions of the invention for the treatment of malignant gliomas. Malignant gliomas are particularly difficult to treat. Approaches employing viral vectors for treatment of malignant glioma have failed to demonstrate

satisfactory infection efficiency (See figures 3A-F of the instant application). This is thought to be due to the histological structure of malignant glioma which is a highly dense tumor, almost completely impermeable to penetration by particles the size of viruses or larger (Shir A, Levitzki A (2001) Gene therapy for glioblastoma: Future perspective for delivery systems and molecular targets. Cell Mol Neurobiol 21: 645–656.).

In light of the fact that viral infection is not an efficient method for killing malignant glioma cells, Applicant submits that is not obvious that the composition of the present invention would be efficient at killing malignant glioma cells, since there is no reason to suppose that the composition of the present invention could penetrate those cells which viruses cannot.

The claims have also been amended to limit the composition to one comprising a specific targeting moiety (EGF), a specific nucleic acid carrier (PEI) and a cytotoxic agent (dsRNA).

Malignant gliomas are characterized by resistance to standard treatment modalities including surgery, radiation therapy and chemotherapy. Accordingly, it is not obvious that malignant gliomas would not be resistant to the cytotoxic effects of dsRNA.

The present composition combines three features which provide it with the potential to cure malignant glioma. These features include:

a) high selectivity and safety, to avoid damage to non-cancerous brain tissue; (b) rapid and efficient cell killing by simultaneous activation of multiple killing mechanisms - see Figure 1 of the present application. The simultaneous activation of multiple killing pathways ensures tumor cell death, even if one or several pathways are inactive; and, (c) inhibition of the growth or killing of neighboring, unexposed tumor cells by cytokine secretion. This "bystander effect" assists in eliminating the tumor before it can re-grow. It also inhibits the growth of any tumor cells that may have a different phenotype from the targeted cells and are not themselves targeted by the treatment, including cancer stem cells.

The finding that targeted delivery of dsRNA results in secretion of cytokines adds to the cytotoxic effect of dsRNA and enables the composition to kill densely packed, unexposed cancer cells such as malignant glioma cells.

Applicant further wishes to point out that a wide selection of nucleic acid

carriers are known in the art – including for example cationic lipids (1,2-Dilauroyl-sn-Glicero-3-Phosphoethanolamine (DLPE), 1,2-Dilauroyl-sn-Glicero-3-Glycerol (DLPG), dioleoyl-1,2-diacyl-3-trimethylammonium-propane (DOTAP) and *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethlylammonium chloride (DOTMA); dimethyldioctadecylammonium (DDAB); 1,2-dilauroyl-sn-glycero-3-ethylphosphocholine (Ethyl PC); 1,2-di-(9Z-octadecenoyl)-3-dimethylammonium-propane and 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Cholesterol) and other synthetic polymers such as poly-L-lysine, poly-Arginine and poly-Histidine. Other exemplary nucleic acid carriers include polyethylenimine, chitosan and dendritic polyaminoamido (PAMAM) dendrimers.

Further a wide selection of malignant glioma targeting moieties are known in the art - including for example hyaluronan targeting the CD44 receptor, antibodies against the neurokinin 1 receptor, ephrinA1 which targets EphA2 and the receptor protein tyrosine phosphatase zeta. multiforme.

Accordingly, selection of the particular combination of EGF, PEI and dsRNA out of the myriad of possible combinations of targeting moiety, cytotoxic agent and nucleic acid carrier known in the literature is also not obvious.

Other combinations of cytotoxic molecules and targeting moieties have been used for the treatment of glioblastoma. Thus, for example Diphtheria toxin linked to EGF has been used for the treatment of astrocytomas and glioblastoma multiforme. However, this molecule shows high toxicity and causes immunodepletion (see for example DAB389EGF fusion protein therapy of refractory glioblastoma Cohen KA, Liu T, Bissonette R, Puri RK, Frankel AE. Curr Pharm Biotechnol. 2003 Feb;4(1):39-49), enclosed herein. Thus, it is not obvious that the presently claimed composition would not show the same effects during the treatment of malignant glioma.

For all the above reasons, Applicant contends that a composition comprising dsRNA, PEI and EGF for the treatment of malignant glioma is not obvious.

In view of the above amendments and remarks it is respectfully submitted that claims 96-99 and 107-108, 110-113 and 115 are now in condition for allowance. Prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

Martin D. Moynihan
Registration No. 40,338

Date: September 24, 2009

Enclosures:

- Petition for Extension (One Month)
- Additional Claims Transmittal Sheet
- Shir et al reference
- Cohen et al reference

$DAB_{389}EGF$ Fusion Protein Therapy of Refractory Glioblastoma Multiforme

Kimberley A. Cohen[1], TieFu Liu[1], Reid Bissonette,[2] Raj K. Puri,[3] and Arthur E. Frankel[1,*]

[1]*Department of Medicine and Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC;* [2]*Ligand Pharmaceutical Corporation, San Diego, CA;* [3]*United States Food and Drug Administration, Bethesda, MD, USA*

Abstract: Primary brain tumors including anaplastic astrocytomas and glioblastoma multiforme are difficult to treat because of their locally invasive nature and chemoradioresistance. Novel therapies are needed. One class of therapeutics is fusion proteins consisting of peptide toxins fused to brain tumor selective ligands. $DAB_{389}EGF$ is a fusion protein composed of the catalytic and translocation domains of diphtheria toxin fused via a His-Ala linker to human epidermal growth factor (EGF). $DAB_{389}EGF$ is selectively toxic to EGF receptor (EGFR) overexpressing cells. Close to half of all high-grade primary brain tumors have EGFR gene amplification and EGFR overexpression. With the use of convection-enhanced delivery (CED), $DAB_{389}EGF$ may be delivered locally at high concentrations to the brain tumor. CED would avoid many of the pharmacologic and toxicologic barriers which have limited effective use of this agent including rapid clearance from the circulation, high anti-diphtheria toxin antibody titers in the blood and toxicities to the liver and kidney. Both cell lines and animal models are available to assess the potential of this agent for brain tumor therapy. Since significant amounts of clinical grade $DAB_{389}EGF$ are available, some careful additional preclinical efficacy work should lead to testing of this agent in patients within the next few years.

HIGH GRADE GLIOMAS

There are 20,000 cases of primary brain tumors/years in the United States [1]. Over 80% of these tumors are infiltrative astrocytomas or glioblastoma multiforme which arise from malignant transformation of astrocytes [2]. There are two peak ages for these tumors--2 years of age (3/100,000 population) and 70 years of age (18/100,000 population). Central nervous system tumors are the most prevalent solid neoplasms of childhood, the second leading cancer-related cause of death in children under 15 years of age, and the third leading cancer-related cause of death in adolescents and adults up to age 35 years old. There are no definitive environmental or genetic risk factors for brain tumors.

Symptoms and signs of intracerebral tumors include headache, nausea, vomiting, personality change, altered psychomotor function up to and including coma, seizures, pituitary endocrine disorders and focal neurological deficits.

Diagnostic tests include magnetic resonance imaging (MRI) with gadolinium-DPTA contrast agent, computerized tomography (CT) with iodine contrast agent, positron emission tomography (PET). Definitive diagnosis requires biopsy and histopathologic examination.

Treatments include corticosteroids to reduce brain swelling, surgical resection, radiotherapy, and chemotherapy. The outcome of patients with high grade gliomas remains dismal with a median survival for newly diagnosed patientsof one year and a median survival for recurrent gliomapatients of six months (Fig. **1**) [3,4]. Chemo-radioresistant tumor cells and local tumor growth are the main causes of death with 90% of recurrences at or adjacent to the site of origin of the disease [5,6]. Novel agents targeting tumor-selective signaling pathways and new methods of delivery have been sought to improve the prognosis in this disease. One new method is convection-enhanced delivery which permits direct delivery of agents to the brain tumor interstitium.

CONVECTION-ENHANCED DELIVERY

The blood-brain barrier reduces the permeability and uptake of most drugs into the central nervous system. Penetration is most inhibited for large molecular weight polar compounds such as proteins. One strategy to overcome this barrier is establishing a pressure gradient during interstitial infusion into the brain. This condition (convection-enhanced delivery or CED) creates a bulk flow which supplements diffusion and leads to achievement of drug concentrations orders of magnitude greater than by systemic administration for large areas of the brain parenchyma [7,8]. CED was pioneered by Oldfield and colleagues at NIH. Catheters are placed in regions of brain tumors and slow rates of infusion of drugs are then administered under pressure at rates of 0.5-5 microliters/minute for one-ten days (Fig. **2**). The technique is particularly effective for proteins. Proteins are not reabsorbed into brain capillaries and thus achieve greater tissue penetration than small molecular weight compounds. CED has been used to deliver directly to brain tumors several fusion proteins including transferrin-CRM107 conjugate, IL4(38-37)-PE38KDEL, and IL13PE38QQR [9-13]. CED with these agents has yielded clinical remissions lasting years in a number of patients. While the currently available brain tumor selective fusion proteins have shown

*Address correspondence to this author at the Hanes 4046, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157, USA; Tel: 336-716-3313; Fax: 336-716-0255;
E-mail: afrankel@wfubmc.edu

1389-2010/03 $41.00+.00

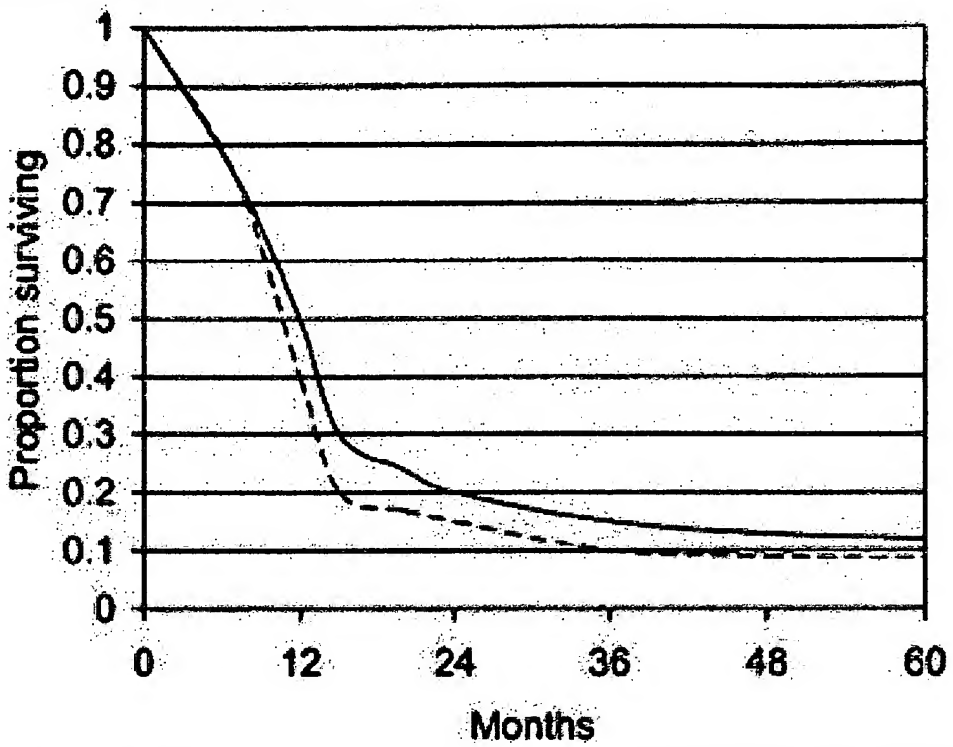

Fig. (1). Overall survival of adult patients with high-grade gliomas.[3] Kaplan-Meier curves. Dotted line is patients treated with radiotherapy alone (n = 1306). Solid line is patients receiving radiotherapy and chemotherapy (n = 1698).

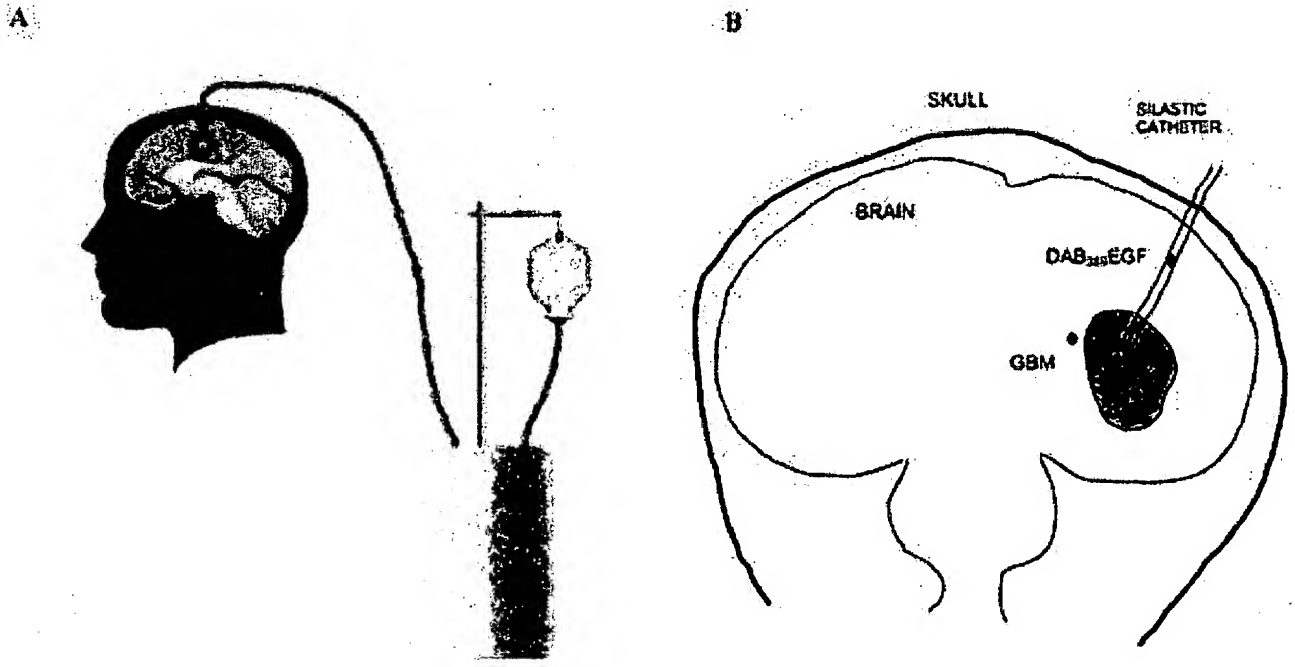

Fig. (2). Diagram of CED. A&B. Silastic-infusion catheters (2.1-mm outer diameter, Pudenz Medtronics, PS Medical, Galeta, CA) are inserted with the tip at a selected site in the tumor using stereotactic guidance through small-twist drill hoes. The proximal ends of the catheters are sewn to the scalp. Catheters and tubing are filled with drug and connected to Medex 2010 micropumps (Medtronic, Minneapolis, MN) filled with drug. These procedures are performed under local and general anesthesia as necessary. Infusion begins immediately or within 24 hours after catheter insertion at 3-5 μL/minute/catheter. The total volume and time vary dependent on tumor size (30 cc to 180 cc). The drug concentration is dose escalated in patient cohorts.

activity, there is a need for additional agents. Transferrin-CRM107 damages normal brain capillaries. IL4(38-37)-PE38KDEL and IL13PE38QQR do not react will all brain tumors.

EPIDERMAL GROWTH FACTOR EXPRESSION BY HIGH GRADE GLIOMAS

To date, protein drugs have been targeted to IL4 receptors, transferrin receptors, and IL13 receptors on malignant gliomas. However, most glioma cells express very few of these receptors on their cell surfaces--usually hundreds to a few thousand/cell. Another target is the epidermal growth factor receptor (EGFR) which is present at about 100,000 molecules/glioma cell [14]. EGFR is an 1,186 amino acid residue transmembrane glycoprotein composed of an N-terminal extracellular ligand binding domain (621 amino acids) , a membrane spanning region (23 amino acids) and a C-terminal intracellular domain (542 amino acids) with tyrosine kinase activity (Fig. 3). The receptor is a phosphoprotein with phosphorylation sites at Thr-654, Thr-669, and Tyr-1173, Tyr-1068 and Tyr-1148, among others. Protein kinase C phosphorylates Thr-654 of the EGFR which accelerates receptor internalization. EGFR ligands include the 53 amino acid single chain polypeptide epidermal growth factor and the structurally related transforming growth factor-α (TGF-α) [15]. EGF binds to EGFR expressed at low levels on normal tissues such as epidermoid and mucosal cells but EGFR is expressed at much higher levels on malignant solid tumors. Over 40% of all glioblastomas have amplification of the EGFR gene (up to 60-fold) and overexpress both EGFR mRNA and protein [16]. EGFR overexpression has been correlated with poorer prognosis in brain tumor patients [17]. EGFR overexpression may be critical for maintenance of the malignant phenotype as glioma cells possess an EGFR/TGF-α autocrine loop which produces constitutive stimulation of pathways important for cell survival, spreading and proliferation [18]. Since EGFR binds the EGF ligand with high affinity and the EGF-EGFR complex internalizes efficiently by receptor-mediated endocytosis, the EGFR is an excellent target for fusion proteins with toxins such as ricin, Pseudomonas exotoxin and diphtheria toxin (DT) [19-21].

Because of prior successful clinical experience with the DT fusion protein targeted to the human interleukin-2 receptor ($DAB_{389}IL2$ or ONTAK) [22], we focused our intention on a DT fusion protein.

DIPHTHERIA TOXIN AND DIPHTHERIA TOXIN ENGINEERING

DT has a 25-residue leader sequence followed by 535 amino acid residues [23]. The mature protein has three

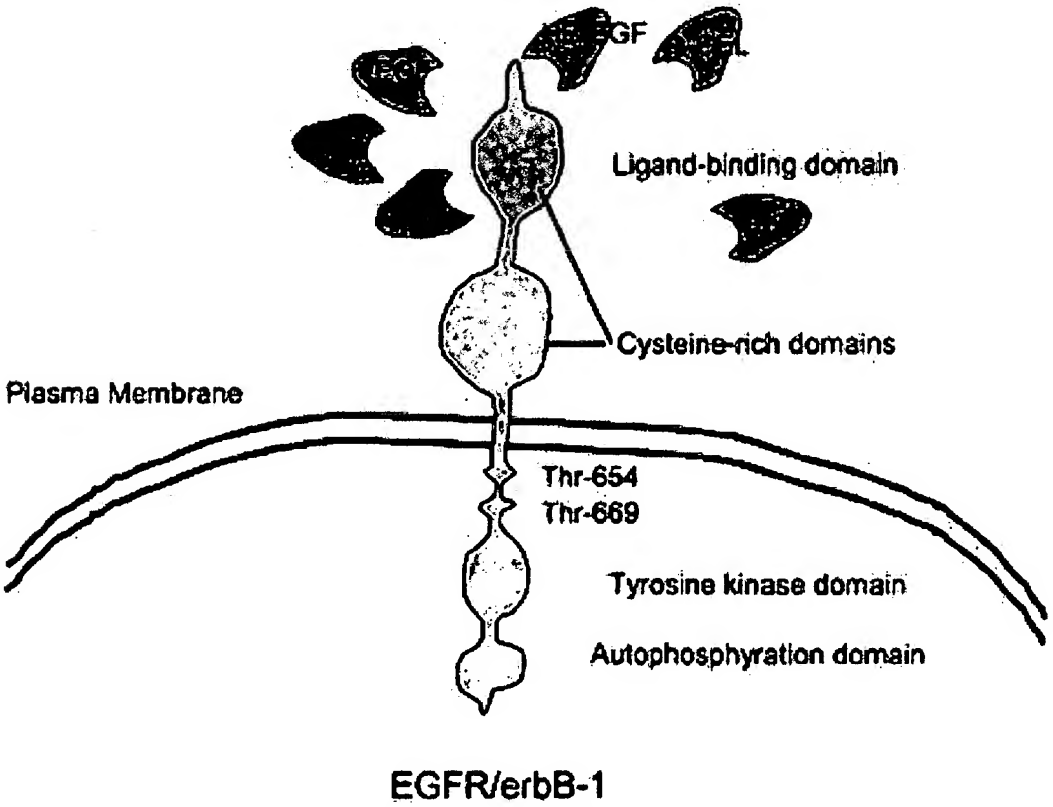

Fig. (3). EGFR model. The cysteine-rich domain is the ligand binding site. Thr-654 is the major protein kinase C target for ligand-induced internalization. Thr-669 is the target for MAP kinase for signal transduction.[18] EGF family member ligands for EGFR include TGF-α (tumor growth factor-alpha), AR (amphiregulin), EGF (epidermal growth factor), HB-EGF (heparin-binding EGF), β-cel (β-cellulin) and Epi (epiregulin) [15].

domains. There is an N-terminal catalytic domain (amino acid residues 1-186), also called the A fragment. This domain is followed by a fourteen amino acid loop bordered by Cys-186 and Cys-201. The loop is arginine-rich, and is a substrate for endosomal furin endoprotease. The second domain is the translocation domain (amino acid residues 187-381) and contains multiple amphipathic helices with two negatively charged amino acid residues (Glu-349 and Asp-352) at a helical hairpin between two of these helices (TH8 and TH9). The translocation domain ends in a flexible spacer (amino acid residues 382-390), which is then connected to a β-sheet rich cell-binding domain (amino acid residues 391-535).

DT binds via residues in the cell-binding domain to cell surface expressed heparin-binding epidermal growth factor-like growth factor precursor (Fig. 4) [24]. After association with CD9 and heparin sulfate proteoglycan, the complex undergoes dynamin and clathrin-dependent endocytosis. Once internalized into early endosomes, DT undergoes furin cleavage at the arginine-rich loop, low pH-induced protonation of the helical hairpin aspartate and glutamate, insertion of the TH8 and TH9 amphipathic helices into the vesicle membrane, unfolding of the catalytic domain, reduction of the disulfide bridge linking the A fragment with the remainder of DT and transfer of the A fragment through the membrane to the cytosol. In the cytosol, the A fragment ADP-ribosylates elongation factor 2 on its diphthamide residue in domain IV. This irreversible modification prevents elongation factor displacement of the tRNA-peptidyl complex from the A site to the P site of the ribosome and halts protein synthesis. Cells then die by lysis or programmed cell death. The prolonged action of a single A fragment in the cytosol is sufficient to inhibit protein synthesis and prevent cell growth and division.

The normal tissue binding function of DT has been removed by genetically replacing the C-terminal receptor binding domain (amino acid residues 391-535) with cell-selective ligands such as melanocyte-stimulating hormone, interleukin-2 (IL2), interleukin-6, gastrin-releasing peptide, and others [25]. These DT fusion proteins have selective and potent killing activity on cells bearing the targeted receptors. The same technique was used to create a DT-EGF chimera.

$DAB_{389}EGF$

DNA encoding $DAB_{389}IL2$ was modified to replace the IL2 sequences with EGF sequences. The final plasmid DNA

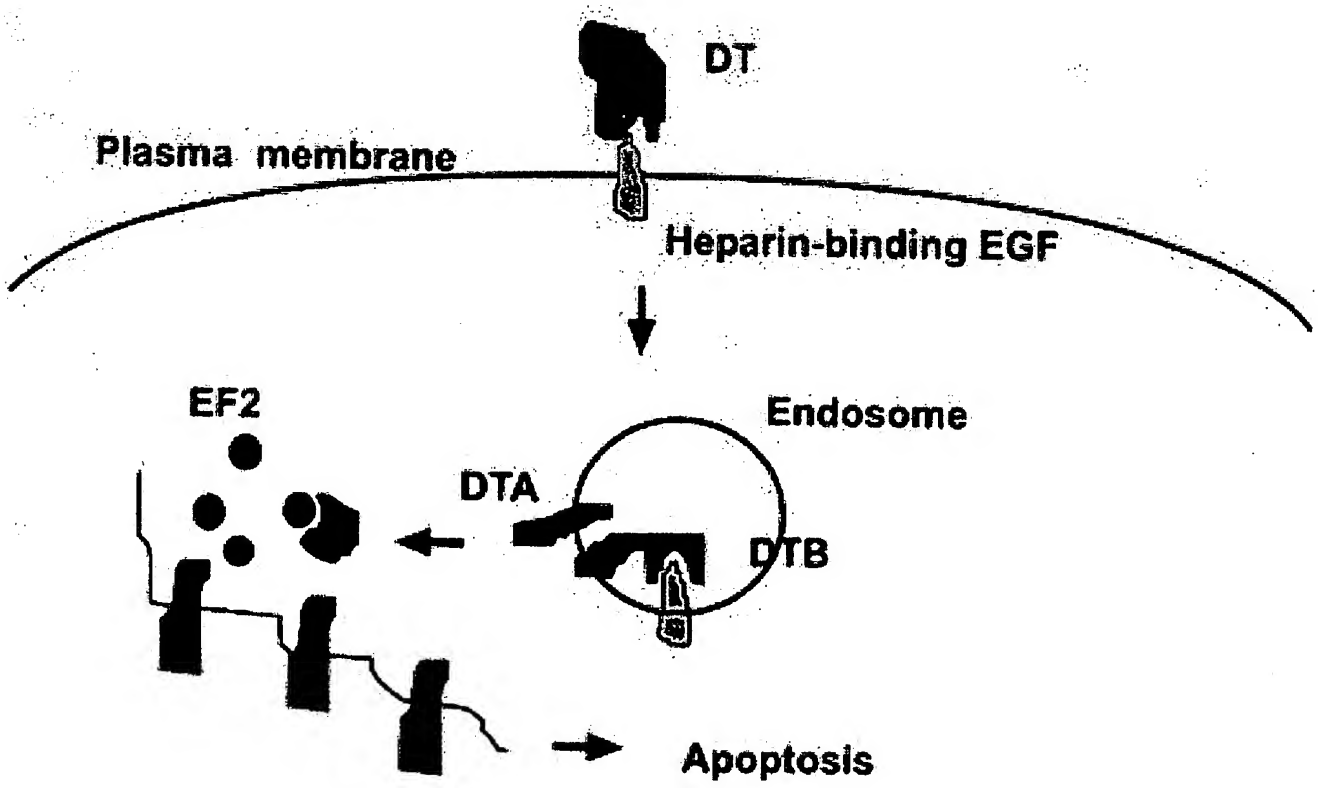

Fig. (4). Steps in DT intoxication of cells. DT binds to heparin-binding EGF-like precursor in association with heparin and CD9. Endocytosis of the DT-DT receptor complex occurs from clathrin-coated pits. Endosomal furin cleaves at arginines in the peptide loop between the catalytic A fragment and DT translocation domains. The endosomal acid pH leads to protonation of a critical aspartate and glutamate facilitating hydrophobic insertion of DT TH8 and TH9 amphipathic helices into the endosomal membrane. Disulfide bond reducing occurs and unfolding of the A fragment. The A fragment then associates with the translocation domain and is transported to the cytosol. The A fragment then refolds and then ADP-ribosylates elongation factor 2. This halts peptide elongation and leads to apoptosis.

was used to transform *E. coli* and protein induced. The recombinant protein contained amino acid residues 1-387 of DT followed by a His-Ala linker followed by amino acids 1-53 of EGF (Fig. **5**) [21]. Initial characterization of the fusion protein showed a molecular weight of 48,522 Da, reactivity with antibodies to DT and EGF on immunoblots, and selective cytotoxicity. EGFR positive human tumor cell lines were killed at picomolar concentrations (Fig. **6**) whereas normal primary tissue cells with few receptors were not killed at nanomolar concentrations. Further analysis showed ADP-ribosylation of elongation factor 2 in sensitive cells. Endosome acidification was necessary as chloroquine treated EGFR positive cells were insensitive to the fusion protein. The affinity of $DAB_{389}EGF$ for the EGFR was reduced 15-30 fold relative to EGF alone. 50 pM $DAB_{389}EGF$ reduced colony formation of EGFR positive tumor cells by greater than a hundred-fold.

To produce drug for preclinical and clinical studies, seedlots of transformed *E. coli* were inoculated into a fermentor. Cultures were grown to mid-logarithmic phase and induced with isopropyl β-D-thiogalactopyranoside (IPTG) to express recombinant protein. The culture was harvested, and cells lysed with a cell homogenizer. Insoluble nclusion bodies containing the protein were collected and washed extensively. The protein was then solubilized with guanidine hydrochloride, diafiltered into weak ionic strength solution for renaturation, and exposed to copper sulfate to trigger disulfide bond formation. Copper was then removed with ethylenediaminetetraacetic acid (EDTA). Protein was purified by DEAE ion exchange chromatography, formulated in phosphate buffered saline pH 7.2 with 1% mannitol and 50 μM EDTA, sterile filtered, vialed and lyophilized. After quality control testing, drug was stored at -20°C until used. Each vial contains 500 μg of $DAB_{389}EGF$, sodium chloride, sodium phosphate, mannitol and EDTA as described above. Drug is prepared by adding 2 mL sterile water and gently stirring. Drug stability studies suggest retention of potency for over five years (Fig. **7** and Table **1**).

PRECLINICAL PHARMACOLOGY AND TOXICOLOGY OF $DAB_{389}EGF$

Rats were administered different doses of $DAB_{389}EGF$ for ten to fourteen days by daily bolus intravenous infusions.

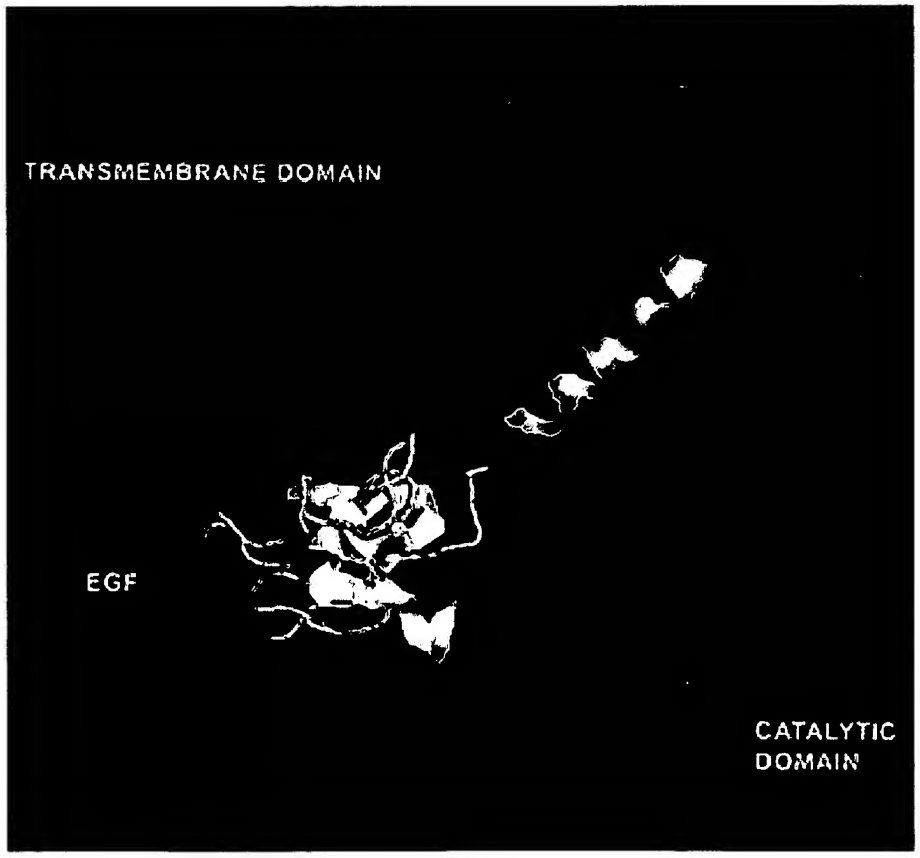

Fig. (5). Ribbon representation of the α-carbon backbone structure of a model of $DAB_{389}EGF$. White is His-Ala-EGF; green is the DT translocation domain; blue is the DT catalytic domain.

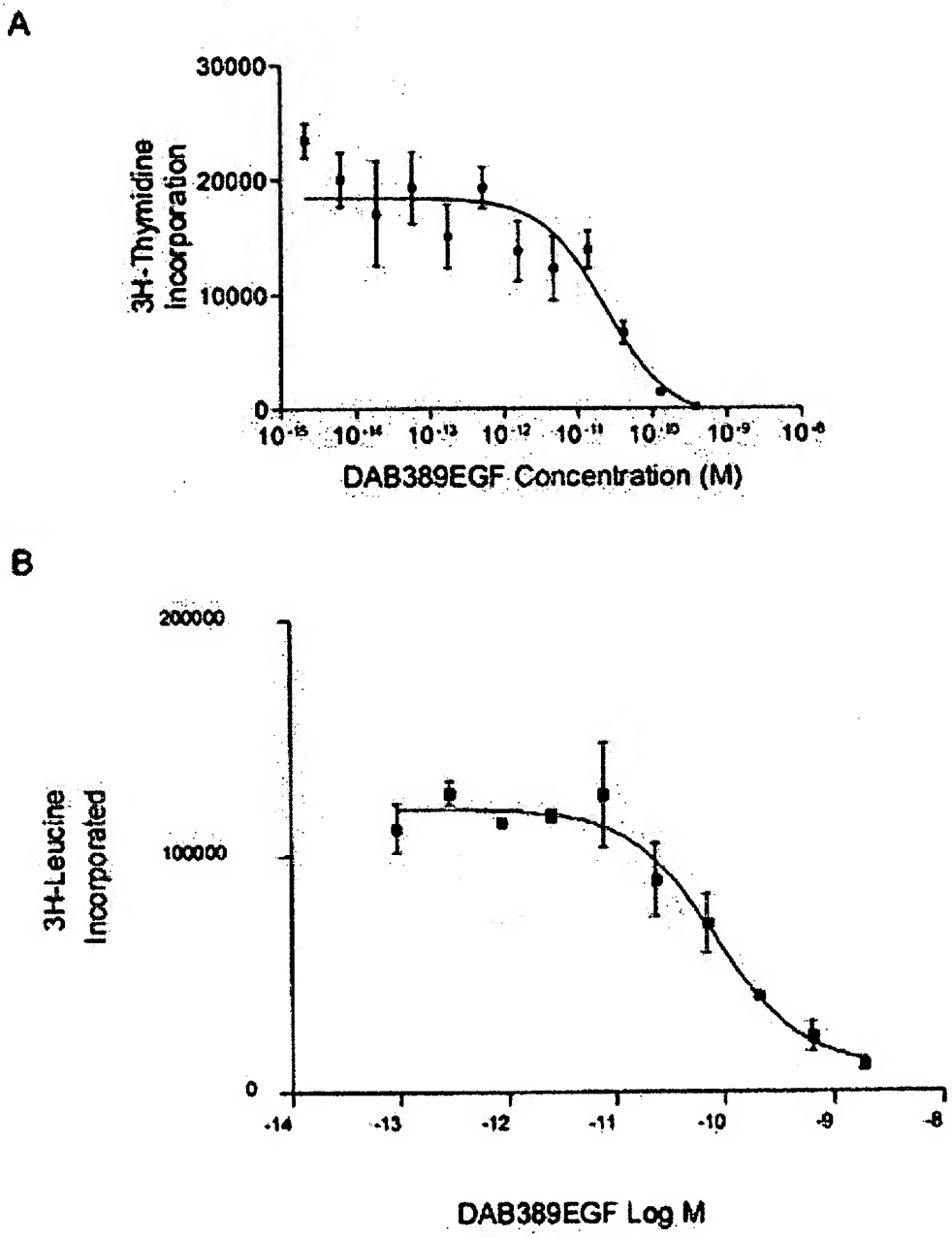

Fig. (6). A431 cell cytotoxicity by $DAB_{389}EGF$ (lot 3E13EB2). Ten thousand cells were incubated 48 hours with the indicated concentrations of fusion protein and then pulsed with either 1 μCi ^{3}H-thymidine (A) or 1 μCi ^{3}H-leucine (B) and harvested onto glass fiber mats with a cell harvestor and counted on a Betaplate scintillation counter. The IC_{50} was 25 pM for proliferation inhibition (A) and 83 pM for protein synthesis inhibition (B).

Dose limiting toxicity (DLT) was renal and hepatic injury and the maximal tolerated dose (MTD) was 30 μg/kg/day. At this dose level, transient elevations in serum creatinine and transaminases as well as increases in urine protein and red blood cells were seen (Table 2). $DAB_{389}EGF$ concentrations were measured in the blood, and the half-life was less than one minute with peak levels of 20 ng/ml for doses of 40 μg/kg/day. ^{35}S-methionine labeled $DAB_{389}EGF$ distributed rapidly, after intravenous infusion of rats, to the liver (61% of injected dose) and kidney (6% of injected dose). Antibody formation to DT occurred within one month in all animals. $DAB_{389}EGF$ was slightly more toxic to cynomolgus monkeys after intravenous infusion with an MTD of 20 μg/kg/day for ten days but with a similar toxicity profile of liver and kidney damage (Table 3).

Nude mice were inoculated subcutaneously with A549 human EGFR positive lung adenocarcinoma cells and treated

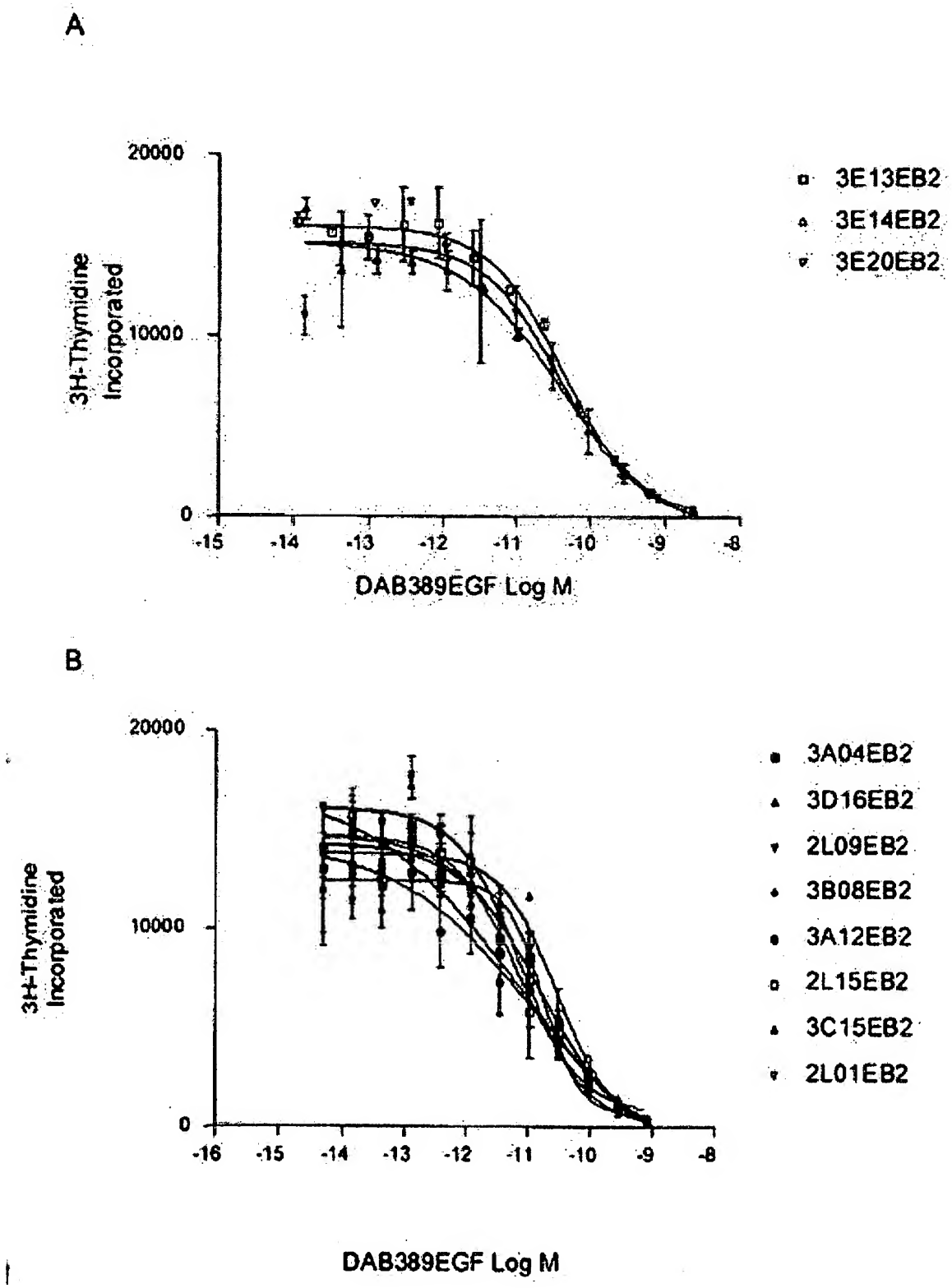

Fig. (7). A431 cell cytotoxicity measured by proliferation inhibition with different lots of $DAB_{389}EGF$. A. Clinical lots. B. All other lots. All experiments were done in duplicate. IC_{50}'s are shown in Table 1.

Table 1. $DAB_{389}EGF$ Batch Potency on A431 Cells*

BATCH	IC_{50} (pM)
3E13EB2	44
3E14EB2	40
3E20EB2	38
3A04EB2	17
3D16EB2	18
2L09EB2	8
3B08EB2	16
3A12EB2	13
2L15EB2	7
3C15EB2	29
2L01EB2	7

*PPD102392B, PPD1030B, 3B16CP2, and 3C12CP2 inactive. The first two batches were early small preliminary batches made in 1992. The latter two batches have inactivating mutations in the DT catalytic and EGF binding domains, respectively. Each batch measured by proliferation inhibition in duplicate. Mean IC_{50} shown. Individual experiments IC_{50}s vary by less than 50%.

starting 24 hours later with $DAB_{389}EGF$ (25 μg/kg/day for ten days). Tumors were 50% smaller in the $DAB_{389}EGF$ treated mice than control vehicle mice by Day 17, and complete tumor regressions were observed in 2/10 $DAB_{389}EGF$ treated mice by Day 14. Nude rats similarly inoculated subcutaneously with A549 cells were treated with one or two five day cycles of $DAB_{389}EGF$ at 50μg/kg/day with the first cycle beginning on Day 0 and the second cycle one week later. As shown in Figure (**8**), $DAB_{389}EGF$ reduced tumor growth by 50% with a single cycle and 70% with two cycles relative to controls.

CLINICAL RESULTS WITH $DAB_{389}EGF$

Three phase I/II clinical studies of intravenous infusions of $DAB_{389}EGF$ have been performed in patients with EGFR positive metastatic malignancies [26]. In the first study, 23 patients received 0.3 - 15 μg/kg/day of $DAB_{389}EGF$ as 30 minute intravenous infusions on five consecutive days. In the second study, 29 patients received 0.3 - 15 μg/kg/day $DAB_{389}EGF$ as 30 minute intravenous infusions on days 1, 8, 9, 15 and 16. Finally, in the third study, 20 patients received 6 - 9 μg/kg/day as 30 minute intravenous infusions on days 1, 3 and 5 every two or three weeks. Lot 3E13EB2 of $DAB_{389}EGF$ was used for each study.

Serum levels of 0 - 50 ng/mL were achieved at the 6 - 9 μg/kg dose levels. A quarter of patients had high anti-DT antibody titers pretreatment. One month later, all patients had high anti-DT antibody titers.

Side effects included asthenia, anorexia, pain, fever, chills, nausea, and vomiting. Laboratory findings included elevated creatinine, elevated transaminases, decreased albumin, decreased hemoglobin and lymphocyte count, increased urine protein, urine red cells and urine white cells.

One patient with non-small cell lung carcinoma had a partial remission lasting six months.

DEVELOPMENT OF $DAB_{389}EGF$ FOR CED OF RECURRENT EGFR POSITIVE GLIOMAS

Many of the properties of $DAB_{389}EGF$ which compromised clinical activity with systemic administration should be beneficial for brain tumor CED therapy. The short half-life of circulating drug and the presence of circulating anti-DT antibodies should reduce side effects but not influence delivery in the brain. The rapid clearance after systemic administration is due to passage through the liver

Table 2. Rat Toxicology Study to Determine the Toxicities Associated with Intravenous Bolus Administration of $DAB_{389}EGF$ for 14 Days: Clinical Chemistry Analysis Day 15 (Mean Value of 10 Rats/Sex/Group)

Male Group	Dose (μg/kg)	BUN mg/dL	Creat mg/dL	Alb g/dL	ALT U/L	AST U/L	ALKP U/L
1	vehicle	13	0.7	4.2	47	144	559
2	10	15	0.7	3.9	67	181	812
3	25	14	0.7	3.9	481	1087	1063
4	40	14	0.7	3.8	70	188	834
Female Group	**Dose (μg/kg)**	**BUN mg/dL**	**Creat mg/dL**	**Alb g/dL**	**ALT U/L**	**AST U/L**	**ALKP U/L**
1	Vehicle	15	0.6	4.1	28	102	294
2	10	16	0.6	4	49	133	429
3	25	15	0.6	3.9	66	185	390
4	40	16	0.6	4.1	41	148	361

Table 3. Cynomolgus Monkey Toxicology Study to Determine the Toxicities Associated with Intravenous Bolus Administration of $DAB_{389}EGF$ for 14 Days: Clinical Chemistry Analysis Day 15 (Mean Value of 3 Monkeys/Group)*

Male Group	Dose μg/kg	BUN mg/dL	Creat mg/dL	Alb g/dL	ALT U/L	AST U/L	ALKP U/L
1	vehicle	22	0.8	3.9	71	32	1664
2	2.5	26	1	3.5	161	78	1757
3	15	33	1.3	3.1	281	90	2025
Female Group	**Dose μg/kg**	**BUN mg/dL**	**Creat mg/dL**	**Alb g/dL**	**ALT U/L**	**AST U/L**	**ALKP U/L**
1	vehicle	23	0.7	3.5	68	26	661
2	2.5	26	0.8	3.3	112	44	1122
3	15	36	0.9	2.7	406	223	1966

*BUN, blood urea nitrogen; Creat, creatinine; Alb, albumin; ALT, alanine transaminase; AST, aspartate transaminase; ALKP, alkaline phosphatase.

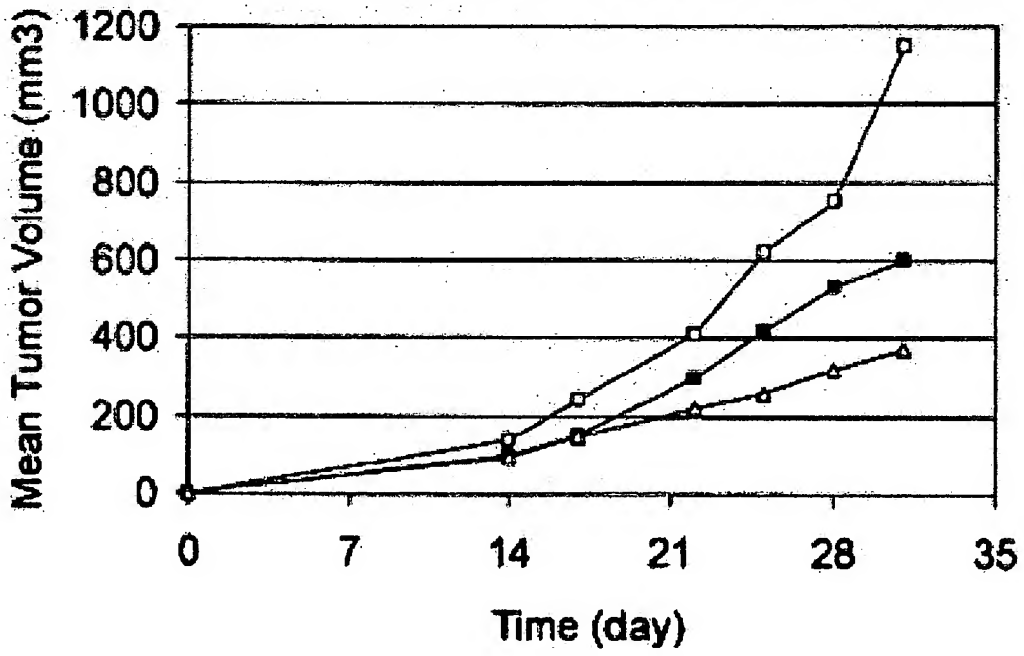

Fig. (8). A549 EGFR positive lung adenocarcinoma tumor volume after subcutaneous implantation in nude rats after $DAB_{389}EGF$ administration. Treatments on day 0 - 4 for first cycle and 7 - 11 for second cycle with 50 μg/kg/day. Empty square, vehicle control; filled square, one cycle; empty triangle, two cycles.

and kidneys. Brain antibody concentrations are much lower than those found in the circulation. Metabolism and toxicities to liver and kidneys should be reduced because very low systemic concentrations of $DAB_{389}EGF$ should occur. Normal tissue toxicities should also be reduced since high concentrations will only be present in the brain and brain tumor. The normal glial, neurons and brain capillaries do not have high EGFR expression. The several thousand vials of clinical use $DAB_{389}EGF$ should be adequate for over a thousand patients since very little drug is needed per patient for CED (estimated at 1 μg/cc tumor volume). Either frozen sections or paraffin sections can be assayed for EGFR with readily available commercial antibodies and immunohistochemistry. In fact, some clinical centers are using EGFR measurements as part of their staging [27]. The availabili-ty of well defined glioma cell lines with high EGFR and sensitivity to $DAB_{389}EGF$ (Fig. 9) and several well-studied animal models also expedites drug development [28]. Finally, the physicians who would be involved both in clinical trials and eventual clinical practice with $DAB_{389}EGF$ for brain tumor CED are a small group of several hundred neurosurgeons. This should expedite both clinical testing and physician education and acceptance for newly approved agents. There are currently 2,963 vials (each containing 0.50 mg) of clinically approved lots of $DAB_{389}EGF$. Based on the current information, $DAB_{389}EGF$ represents an exciting new therapeutic for some cancer patients with an otherwise dismal prognosis.

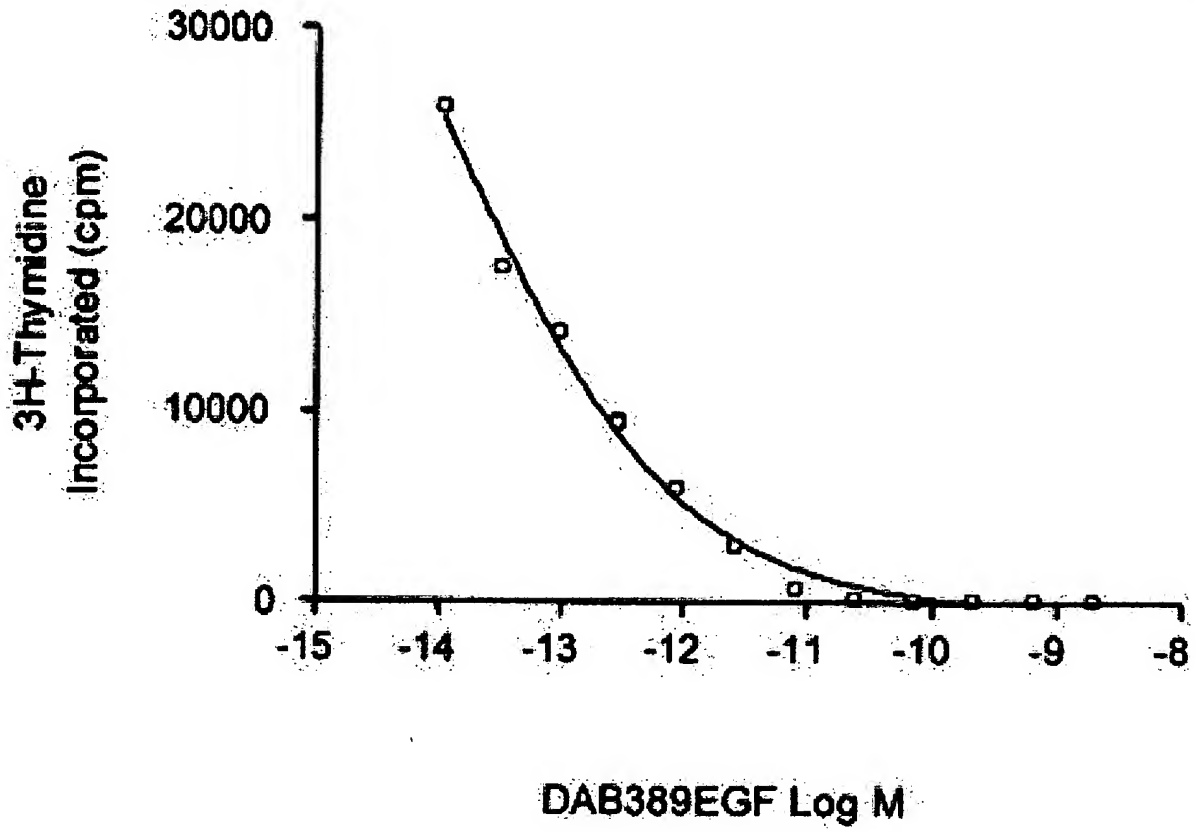

Fig. (9). U373MG human glioma cell proliferation inhibition by $DAB_{389}EGF$ (lot 3E13EB2). Performed identically to Figure 6 above. The IC_{50} was 0.01 pM.

REFERENCES

[1] Greenlee, R.T., Hill-Harmon, M.B., Murray, T., Thun, M. (2001) *CA Cancer J. Clin.*, **51**, 15-36.

[2] Polednak, A.P., Flannery, J.T. (1995) *Cancer 75,* **Suppl 1,** 330-337.

[3] Stewart, L.A. (2002) *Lancet*, **359**, 1011-1018.

[4] Wong, E.T., Hess, K.R., Gleason, M.J., Kaeckle, K.A., Kyritsis, A.P., Prados, M.D., Levin, V.A., Yung, W.K.A. (1999) *J. Clin. Oncol.*, **17**, 3572-2578.

[5] Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S.N., Hidalgo, O.F., Vanaclocha, V., Baylin, S.B., Herman, J.G. *New Engl. J. Med.*, **343**, 1350-1354, 2000.

[6] Gaspar, L.E., Fisher, B.J., Macdonald, D.R., LeBer, D.V., Halperin, E.C., Schold, S.C., Cairncross, J.G. (1992) *Intl. J. Rad. Onc. Biol. Phys.*, **24**, 55-57.

[7] Bobo, R.H., Laske, D.W., Akbasak, A., Morrison, P.F., Dedrick, R.L., Oldfield, E.H. (1994) *Proc. Natl. Acad. Sci.*, **91**, 2076-2080.

[8] Laske, D.W., Morrison, P.F., Lieberman, D.M., Corthesy, M.E., Reynolds, J.C., Stewart-Henney, P.A., Koong, S.S., Cummins, A., Paik, C.H., Oldfield, E.H. (1997) *J. Neurosurg.*, **87**, 586-594.

[9] Laske, D.,W., Youle, R.J., Oldfield, E.H. (1997) *Nature Med.*, **3**, 1362-1368.

[10] Oldfield, E.H., Broaddus, W.C., Bruce, J., Task, T., Laske, D.W., McDonald, J., Patel, S.J., Weingart, J.D., Wharen, R.E., Youle, R.J. (2000) *Proc. Am. Assoc. Neurol. Surgeons,* **18**, 94-95.

[11] Rand, R.W., Kreitman, R.J., Patronas, N., Varricchio, F., Pastan, I., Puri, R.K. (2000) *Clin. Cancer Res.*, **6**, 2157-2165.

[12] Weingart, J., Grossman, S.A., Bohan, E., Fisher, J.D., Strauss, L., Puri, R.K. (2001) *Proc. World Fed. Neuro-Oncology*, Nov. 15 – Nov. 17, 2001 Washington, DC.

[13] Prados, M., Lang, F., Strauss, L., Fleming, C., Aldape, K., Kunwar, S., Yung, W.K.A., Husain, S.R., Chang, S.M., Gutin, P., Raizer, J., Piepmeier, J., Berger, M., Puri, R.K. (2001) *Proc. World Fed. Neuro-Oncology,* Nov. 15 – Nov. 17, 2001 Washington, DC.

[14] Wong, A.J., Bigner, S.H., Bigner, D.D., Kinzler, K.W., Hamilton, S.R., Vogelstein, B. (1987) *Proc. Natl. Acad. Sci. USA,* **84**, 6899-6903.

[15] Yarden, Y. (2001*Eur. J. Cancer,* **37** Suppl. 4, S3-S8.

[16] Sauter, G., Maeda, T., Waldman, F.M., Davis, R.L. Feuerstein, B.G. (1996) *Am. J. Pathol.*, **148**, 1047-1053.

[17] Chakravarti, A., Delaney, M.A., Noll, E., Black, P.M., Loeffler, J.S., Muzikansky, A., Dyson, N.J. (2001) *Clin. Cancer Res.*, **7**, 2387-2395.

[18] Tang, P., Steck, P.A., Yung, W.K. (1997) *J. Neuro-Oncology*, **35**, 303-314.

[19] Cawley, D.B., Herschman, H.R., Gilliland, D.G., Collier, R.J. (1980) *Cell*, **22**, 563-570.

[20] Kunwar, S., Pai, L.H., Pastan, I. (1993) *J. Neurosurg.*, **79**, 569-576.

[21] Shaw, J.P., Akiyoshi, D.E., Arrigo, D.A., Rhoad, A.E., Sullivan, B., Thomas, J., Genbauffe, F.S., Bacha, P., Nichols, J.C. (1991) *J. Biol. Chem.*, **266**, 21118-21124.

[22] Olsen, E., Duvic, M., Frankel, A., Kim, Y., Martin, A., Vonderheid, E., Jegasothy, B., Wood, G., Gordon, M., Heald, P., Oseroff, A., Pinter-Brown, L., Bowen, G., Kuzel, T., Fivenson, D., Foss, F., Glode, M., Molina, A., Knobler, E., Stewart, S., Cooper, K., Stevens, S., Craig, F., Reuben, J., Bacha, P., Nichols, J. (2001) *J. Clin. Oncol.*, **19**, 376-388.

[23] Brooke, J.S., Cha, J.H. (2000) *Biochem. Biophys. Res. Commun.*, **275**, 374-381.

[24] Falnes, P.O., Sandvig, K. (2000) *Curr. Opin. Cell Biol.*, **12**, 407-413.

[25] vanderSpek, J.C., Murphy, J.R. (2000) *Methods Enzymol.*, **327**, 239-249.

[26] Theodoulou, M., Baselga, J., Scher, H., Dantis, L., Trainor, K., Mendelsohn, J., Howes, L., Elledge, R., Ravdin, P., Bacha, P., Bradt-Sarif, T., Osborne, K. (1995) *Pros. ASCO*, **14**, 480.

[27] Etienne, M.C., Formento, J.L., Lebrun-Frenay, C., Gioanni, J., Chatel, M., Paquis, P., Bernard, C., Courdi, A., Bensadoun, R.J., Pignol, J.P., Francoual, M., Grellier, P., Frenay, M., Milano, G. (1998) *Clin. Cancer Res.*, **4**, 2383-2390.

[28] Engebraaten, O., Hjortland, G.O., Juell, S., Hirschberg, H., Fodstad, O. (2002) *Intl. J. Cancer*, **97**, 846-852.

Cellular and Molecular Neurobiology, Vol. 21, No. 6, December 2001 (© 2002)

Commentary

Gene Therapy for Glioblastoma: Future Perspective for Delivery Systems and Molecular Targets

Alexei Shir[1,2] and Alexander Levitzki[1]

Received September 30, 2001; accepted October 24, 2001

INTRODUCTION

Malignant gliomas, the most common subtype of primary brain tumors, are aggressive, highly invasive, and neurologically destructive tumors. These tumors are considered to be among the deadliest of all human cancers. In its most aggressive form, glioblastoma (GBM), median survival ranges from 9 to 12 months. Despite several decades of technological advances in neurosurgery and radiation therapy there has been no significant change in the overall statistics.

Malignant gliomas are attractive targets for local gene therapy because of their complete localization into the central nervous system and absence of remote metastases. In this summary we shall review current molecular strategies used to treat gliomas with special emphasis on gene therapy approaches. Detailed reviews can be consulted (Alemany *et al.*, 2000; Bansal and Engelhard, 2000; Fueyo *et al.*, 1999; Gupta, 2000; Karpati *et al.*, 1999). These strategies include

1. Cell cycle control or inducing apoptosis
2. Suicide gene therapy
3. Immunogene therapy
4. Antiangiogenesis
5. Oncolytic viruses
6. Cancer specific activation of PKR

[1] Unit of Cellular Signaling, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel.
[2] To whom correspondence should be addressed at Unit of Cellular Signaling, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Givat Ram, Jerusalem 91904, Israel; e-mail: alexeis@cc.huji.ac.il.

0272-4340/01/1200-0645/0

It should be stated at the outset that while these approaches have proved successful in vitro and in animal models, none of these strategies have met with success in clinical trials. Possible reasons for this clinical failure will be discussed here as will be ideas for improving gene therapy protocols for glioblastomas.

STRATEGIES FOR GENE THERAPY OF GLIOMA

In Table I we summarize the gene therapy protocols, currently under study for the treatment of glioblastomas.

Cell Cycle Control and Apoptosis

p53 is an antiproliferative and proapoptotic protein that suppresses transformation either by inducing apoptosis or by blocking cell cycle progression. Mutations of the p53 gene are the hallmarks of approximately 50% of all cancers and are present in more than 30% of astrocytomas. Delivery of the p53 gene to cancer cells could thus be of therapeutic benefit. It is currently being tested in clinical trials. In gliomas, p53-transfer strategies may be insufficient to control tumor growth because many gliomas already express wild-type p53. Furtheremore, the majority of tumors that harbor mutant p53 possess a subpopulation of cells that express wild-type p53 protein and overexpress the Cdk2 inhibitor p21 protein, which causes resistance to p53-mediated apoptosis.

Another molecular target utilized is E2F-1, which appears to be an even more powerful tumor suppressor gene. The E2F-1 protein promotes apoptosis in several systems, both alone and in association with p53 (Gomez-Manzano *et al.*, 1999). Preliminary studies suggest that E2F-1 may be more effective than p53 in glioma cells, since it is able to induce apoptosis in p53-resistant cells (Fueyo *et al.*, 1999). The encouraging preliminary results obtained from the transfer of E2F-1 to glioma in vitro and in animal models should boost clinical trials using intratumoral transfer of E2F-1 either alone or combined with p53, for glioma treatment. The disadvantages

Table I. Gene Therapy Protocols for Glioblastoma

General strategy	Method	References
Cell cycle control or inducing apoptosis	Transfer of p53, RB, P16, E2F-1	Reviewed in Fueyo *et al.*, 1999
Suicide genes and enzyme-prodrug systems	HSV-TK+ganciclovir, Cytosine-deaminase+5-fluorocytosine	Rainov 2000, Ichikawa *et al.*, 2000
Enhancement of the immune system by immunogene therapy	TGF/IL2, IL4, IL2, IFN, HLA	Reviewed in Parney *et al.*, 2000
Antiangiogenesis	Antisense VEGF, Endostatin	Puduvalli and Sawaya, 2000
Oncolytic viruses	HSV-1, ONYX-15, Reovirus	Fueyo *et al.*, 1999; Wilcox *et al.*, 2001
Tumor-specific activation of PKR	AS RNA complementary to Δ(2–7) EGFR mRNA	Shir and Levitzki, 2001

of this molecule are its potential toxic effects on normal cells and possible oncogenic potential, since E2F family proteins facilitate the expression of cell cycle promoting genes (Fueyo *et al.*, 1999; Gomez-Manzano *et al.*, 1999).

Transfers of other tumor suppressor genes such as Rb or p16 to gliomas do not have any significant antiproliferative effect (Fueyo *et al.*, 1999).

One of the abnormalities most frequently found in glioblastomas is the deletion of MMAC1 phosphotase in chromosome 10 (PTEN). The tumor suppressor PTEN is actually, PIP3 phosphatase, the negative regulator of PDK1 and Akt/PKB. Transfer of MMAC1 to cancer cells can suppress tumorigenesis through its ability to regulate cellular differentiation and anchorage-independent growth (Cheney *et al.*, 1999; Wick *et al.*, 1999). Moreover, since MMAC1 is inactivated in the latest stages of glioma progression it may play a role in angiogenesis or invasiveness (Wen *et al.*, 2001).

Suicide Gene Therapy

This strategy involves the transfer of an enzyme-encoded gene, capable of converting a protoxic drug to its toxic form, into the tumor cells. The herpes simplex virus (HSV) thymidine kinase gene converts the non-toxic nucleoside analog, ganciclovir, into its phosphorylated form that kills dividing cells by incorporating itself into DNA molecules in the process of synthesis, thus blocking their elongation. In this way glioma cells transfected by the HSV thymidine kinase gene can be killed by administration of ganciclovir. An important advantage of this suicide system is the "bystander effect" whereby uninfected cells are affected by the production of the cytotoxic drug in neighboring infected cells. The thymidine-kinase–ganciclovir approach is currently being used in several clinical trials (Table I).

Another suicide system is the cytosine-deaminase–5-fluorocytosine system. Here, cytosine deaminase, which converts 5-fluorocytosine to 5-fluorouracil within the cancer cells, is used. The advantage to this system is its broader bystander effect (Ichikawa *et al.*, 2000).

Immunogene Therapy

This strategy includes several approaches:

a. Presentation of Tumor-Rejection Antigens by Antigen-Presenting Cells: Autologous antigen-presenting cells can be harvested by mobilization from a patient's blood or from biopsy specimens of the brain tumor. The cells can then be expanded in vitro with cytokines. To generate antitumor immune response, antigen-presenting cells are transduced with tumor antigen DNA or messenger RNA coding, which is then injected into the patient.
b. Transfer of cytokine encoding genes to tumor cells (Table I): In this approach tumor cells are transduced with a vector which expresses a cytokine such as IL-4 that stimulates the general immune response of the host.

c. Expression of co-stimulatory molecules on the surface of tumor cells: This involves the transfer of costimulatory molecules, such as HLA, into glioma cells. This strategy aims to enhance the expression of tumor-specific antigens on the cell surface and/or activate T-cell responses against the tumor.

Anti-Angiogenic Therapy

Neovascularization is a major feature of glioblastomas (Maher *et al.*, 2001). Angiogenesis activators are extremely important in tumor growth, as reflected by the fact that neovascularization must occur for solid tumors to grow beyond a diameter of 2–3 mm (Goldbrunner *et al.*, 2000). One of the molecules that regulates this process is the vascular endothelial growth factor (VEGF). VEGF mRNA is overexpressed in the highly vascularized glioblastoma multiform (Maher *et al.*, 2001). It has been demonstrated that the transfection of antisense-VEGF–complementary-DNA as well VEGF antisense RNA encoding vectors result in down-regulation of the endogenous VEGF and inhibits growth of gliomas in mice (Sasaki *et al.*, 1999; Zheng *et al.*, 2000). A similar effect was observed upon the local delivery of the angiogenesis inhibitor endostatin (Read *et al.*, 2001). However, this strategy has a cytostatic effect. It is effective in inhibiting tumor growth but not in actually eliminating them. For maximal efficacy antiangiogenesis should be utilized in combination with other therapeutic modalities.

Tumor Specific Activation of PKR

Activated double-stranded (ds) RNA-dependent protein kinase-PKR is a potent growth inhibitory protein that is generally activated in virally infected cells, inducing them to die (Jagus *et al.*, 1999). If achieved, selective activation of PKR in cancer cells could prove a powerful means of treating cancer. Activation of PKR involves two molecules binding in tandem to dsRNA and subsequently phosphorylating each other in an intermolecular event (Wu and Kaufman, 1997). PKR exhibits differing affinities for dsRNAs of different sizes and it has been demonstrated that molecules shorter than 30 base pairs (bp) fail to bind stably and do not activate the enzyme. Molecules longer than 30 bp both bind and activate the enzyme, with an efficiency that increases with increasing chain length, reaching a maximum at about 85 bp (Manche *et al.*, 1992). Many tumor cells, including gliomas, express mutated genes containing deletions or chromosomal translocations that produce unique sequences. About 50% of glioblastomas express constitutively active truncated EGF receptor, Δ(2–7) EGFR (Nishikawa *et al.*, 1994). Antisense RNA of a specific length, complementary to fragments flanking the deletion/translocation, can produce a dsRNA molecule of sufficient length to activate PKR and induce cell death, upon hybridization with mutated, but not wild type, mRNA (Fig. 1). We utilized the U87MGΔEGFR cell line that expresses Δ(2–7) EGFR, as a model to examine this hypothesis. Expression of a 39-nucleotide-long antisense RNA complimentary to the unique exon 1 to 8 junction, causes selective death of cells harboring the truncated EGFR both in vitro and in vivo but do not affect cells expressing wild type EGFR (Shir and Leitzki, 2001). This dsRNA killing strategy can be implemented for the treatment of a variety of cancers, which express unique RNA species.

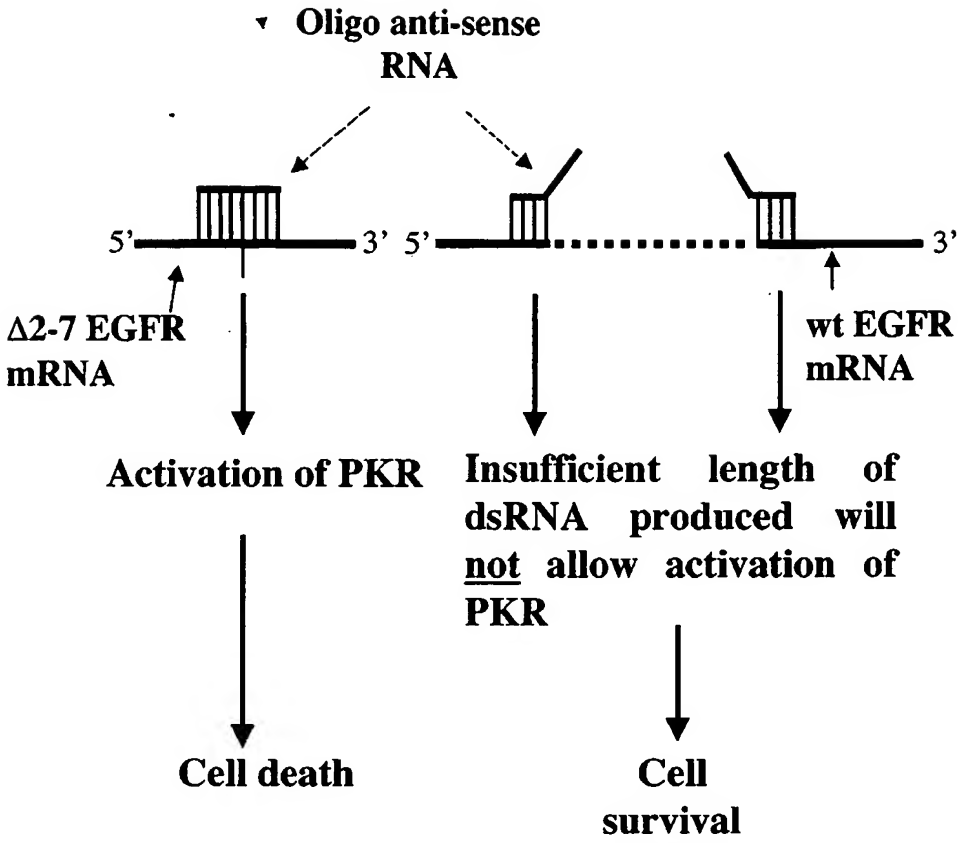

Fig. 1. Mechanism of specific activation of PKR in U87MGΔEGFR cells.

DELIVERY SYSTEMS

Table II summarizes the gene therapy delivery systems for brain tumors.

The ideal gene therapy system for glioblastoma should conform to the following principles. It must kill or infect cancer cells only (preferably both). It should not cause any immunotoxic reaction and it should be pharmacologically controllable. It must also kill all cancer cells within a short period of time, either by infecting each and every cancer cell or through a broad bystander effect.

Despite enormous advances in the development of gene delivery systems there is still no ideal gene therapy vector. The advantages and disadvantages of currently used gene delivery systems have been well reviewed (Alemany *et al.*, 1999; Bansal and Engelhard, 2000, Table II). Currently used systems are very efficient in vitro and in vivo when coinjected simultaneously with the tumor cells. However these systems become far less efficient when used against established tumors, which is of course the clinically relevant model. It is likely that the low in vivo infection efficiency of the vectors is connected to the histological structure of the glioblastomas. These are very solid tumors, which are almost completely impermeable to diffusion of big particles such as viruses.

Table II. Characterization of Delivery Systems

Delivery system	Integration	Cell replication required	In vivo infection efficiency	Immune and general toxicity	Targeting	Control
Retroviruses	**Yes**	Yes	+	−	**Possible magnetic**	**Reversible inactivation**
Retrovirus-producing packaging cell line	**Yes**	Yes	++	++	**Same**	**Same**
RD adenovirus	No	**No**	++	++	**Possible by bispecific antibodies**	No
RC adenovirus	No	**No**	+++++	++++	**Same**	No
RD herpes virus	No	**No**	++	+++	No	**Ganciclovir**
RC herpes virus	No	**No**	+++++	+++++	No	**Same**
Reovirus	No	**No**	+++++	++++	No	No
Lentiviruses	**Yes**	**No**	++	−	**Possible magnetic**	No
Lentivirus-producing packaging cell line	**Yes**	**No**	+++	++	**Same**	**Tet**
Irradiated, encapsulated lentivirus-producing packaging cell line	**Yes**	**No**	++++	−	**Same**	**Same**

Note. RC: replication competent; RD: replication deficient. Words in bold represent advantages of the system. Underlined words represent features that are new or currently under development.

Thus only the cells directly exposed to the vector undergo infection. When a vector is injected systemically or close to the tumor, only the superficial tumor cells are infected. When the vector is injected directly into the center of the tumor, only the cells in the center and the superficial cells are infected. Since the ratio between the number of superficial cells to the number of total cells of the tumor decreases quickly with increased tumor volume, infection efficiency is very low even in small tumors (less than 1%). Several approaches can be used in order to overcome these obstacles.

Oncolytic Viruses

Oncolytic viruses reproduce themselves in cancer cells and subsequently kill the initially infected cells by lysis. They then proceed to infect adjacent cells thus repeating the cycle. This is the most efficient system but it is also the most dangerous. In experiments so far conducted at least 3 oncolytic viruses have proved suitable for glioma treatment in animal models.

Herpes Simplex Virus

Genetically engineered herpes simplex viruses have proved efficient in the elimination of glioma cells in vitro and in vivo (Mineta *et al.*, 1995). Since these viruses are highly toxic and may induce encephalitis in human beings, current research is focused on the generation of genetically altered viruses with low virulence to normal cells. Another advantage of this virus is its sensitivity to ganciclovir. Ganciclover can eliminate the virus if it spreads to normal cells.

Replication-Competent Adenovirus

The replication-competent adenovirus system is based on the fact that adenovirus needs a specific protein of the E1B virus to replicate in the host cells. The E1B protein binds and inactivates the p53 protein and allows the cell to enter into S phase and synthesize the viral proteins necessary for viral replication. An E1B-mutated adenovirus that is incapable of replicating in wild-type p53 cells but is able to replicate and kill mutant TP53 cancer cells was designed and generated. Injection of mutant adenovirus into glioma tumors grown in mice led to complete tumor regression (Bischoff *et al.*, 1996). One disadvantage of this system is the high heterogeneity of gliomas that express wild-type and mutant p53 cells inside the same tumor. It has been shown that this vector can still replicate, albeit at a low rate, in cells possessing functional p53. The selectivity of this vector can be enhanced by directing the vector to cancer cells through bispecifc antibodies directed at cancer-specific antigens and the fiber of the vector (Haisma *et al.*, 2000). The neutralizing immune response of the host against this adenovirus is the principal barrier to successful therapy in humans.

Reovirus

Reovirus is a naturally occurring oncolytic virus that requires activated Ras-signaling pathways of tumor cells for its replication (Wilcox *et al.*, 2001). Ras pathways

are activated in most malignant gliomas via upstream signaling by receptor tyrosine kinases. It has been shown that reovirus efficiently destroys established human glioma models in nude mice (Wilcox *et al.*, 2001). However, it also causes strong toxic reaction in these animals, necessitating careful research of the virus properties in immunocompromised patients. The neutralizing immune response of the host against this virus could also be a significant obstacle to therapy for humans.

Replication Defective Vectors and Replication Defective Vector-Producing Packaging Cells

Despite being less efficient, replication-defective vectors are still much safer than the replication-competent vectors (Table II). Therefore, by increasing efficiency of the replication-defective system it may be possible to reach an optimal compromise between safety and efficacy.

The development of lentiviral vectors with multiple safety features (Naldini and Verma, 2000), have proven the most effective of all replication defective vectors and therefore constitute a major step toward achieving this compromise. Lentiviruses, in contrast to murine leukemia retroviruses, developed a mechanism for active nuclear import of the preintegration complex of the virus (Gallay *et al.*, 1995). This allows for far more efficient transduction of both dividing and nondividing cancer cells. Lentiviruses integrate their genome into the host genome, further ensuring the death of all cancer cells. This feature is absent in adenoviral vectors, and as a result it is often necessary to administer multiple injections of adenoviral vectors that can induce immunotoxic reaction and immune neutralization of the vectors. Lentiviral vectors seem to be especially useful for cancer-specific activation of PKR strategy described above. Further enhancement of the vector for this strategy was performed in our laboratory by introducing powerful U6 promoter into the vector (Shir and Levitzki, 2001).

Selectivity of the lentiviral vectors could be enhanced in the future by magnetic targeting. A method for in vitro magnetic targeting of retroviral vectors was recently developed (Hughes *et al.*, 2001). This method is based on the hypothesis that retroviruses may be captured using antibodies directed against murine fibronectin. It is possible to concentrate captured PG13-derived retrovirus through applying a magnetic field to streptavidim paramagnetic particles (PMPS) conjugated with polyclonal antimurine fibronectin antibodies. Magnetic capturing of retroviral vectors enables production of what are in effect "infectious, paramagnetic, retroviral vector particles," which can then be magnetically guided to the desired location for infection. If a specific shape cut from magnetic sheeting is placed underneath a subconfluent culture of HeLa cells, it can both attract and retain the retrovirus and direct it to primarily infect a specific area, determined by the position of the magnet. Though the magnetic field is weak it is extremely effective in directing retroviral infection in vitro (Hughes *et al.*, 2001). Efficient in vivo targeting is, however, a much more complicated issue requiring sophisticated tools. Small permanent magnets can only be used at accessible sites, and ex vivo generated electromagnetic fields can only focus at one particular site at a time. As with all targeting, the inhibition of infection of those sites not targeted is a major problem; for this reason, reversible inactivation

of retroviral vectors (Pandori and Sano, 2000) is an exciting and complementary development.

This strategy involves a method whereby the infectivity of a retroviral vector is neutralized by treating viral particles with a photocleavable biotinylation reagent. These modified viral vectors possess little to no infectivity for target cells as long as they are not exposed to long-wavelength UV light that activates the infectivity of the viral vector (Pandori and Sano, 2000). Localization of reversibly inactivated retroviral vectors to specific tissues, organs, and metastases followed by localized photoactivation of infection may increase specificity in targeting. Although both these strategies were developed for a murine leukemia-virus–based vector, it is in principle possible to apply both strategies to lentiviral vectors. New developments in electronics may in the near future allow for the construction of small tumor-implantable devices that are controllable or programmable, for creating a magnetic field and UV light at a particular time point.

Injection of cells producing replication-defective virus is much more efficient than injecting high amounts of viruses as continuous release of lower amounts of vectors may overcome the problems of low exposure of cancer cells to vectors described above. A recently developed lentivirus-producing packaging cell line (Xu *et al.*, 2001) could be extremely useful for this strategy, especially as release of the vector can be controlled by tetracycline (Xu *et al.*, 2001). The disadvantages of this system includes possible dissemination of the cells, immunotoxic reactions and immune neutralization. To eliminate these disadvantages we are currently developing technology for encapsulating the irradiated nonreplicative packaging cells into alginate microcapsules. A technique for the treatment of glioblastomas on the basis of local delivery of the antiangiogenic protein endostatin from genetically engineered cells encapsulated in ultrapure sodium alginate was recently described (Read *et al.*, 2001). Alginate consists of L-guluronic and D-mannuronic acid, which in the presence of divalent cations forms an extended gel network, in which cells reside and remain immunoisolated when implanted into the rat brain (Read *et al.*, 2001). The encapsulation technique may be used for many different cell lines engineered to potentially interfere with the complex microenvironment in which tumor and normal cells reside. The viral vector is significantly bigger than a single protein, so permeability remains to be determined. However if successful, this approach could prove very efficient especially when the virus-produced capsules are introduced after removing the bulk of the tumor surgically. The vector generated in the capsules should eliminate the remaining cancer cells or nonremovable micrometastases (Fig. 2) that go on to proliferate and generally kill the patient within a year of initial surgery (Haroun and Brem, 2000; Hochberg and Pruitt, 1980).

It should be noted that all gene therapy systems must kill cancer cells faster than the cells proliferate. This could be extremely difficult to achieve after the tumors have reached a certain size as only a small percentage of the cells are exposed to the vector. The time needed for a vector to infect and kill the superficial cells and make way for the next level of cells may be significantly longer than that needed for cancer cells to replicate themselves. Therefore, cancer cells underlying the cells exposed to the vector can easily compensate for the killing of exposed cells, especially if there are large numbers of them, as in relatively large tumors. Therefore development of

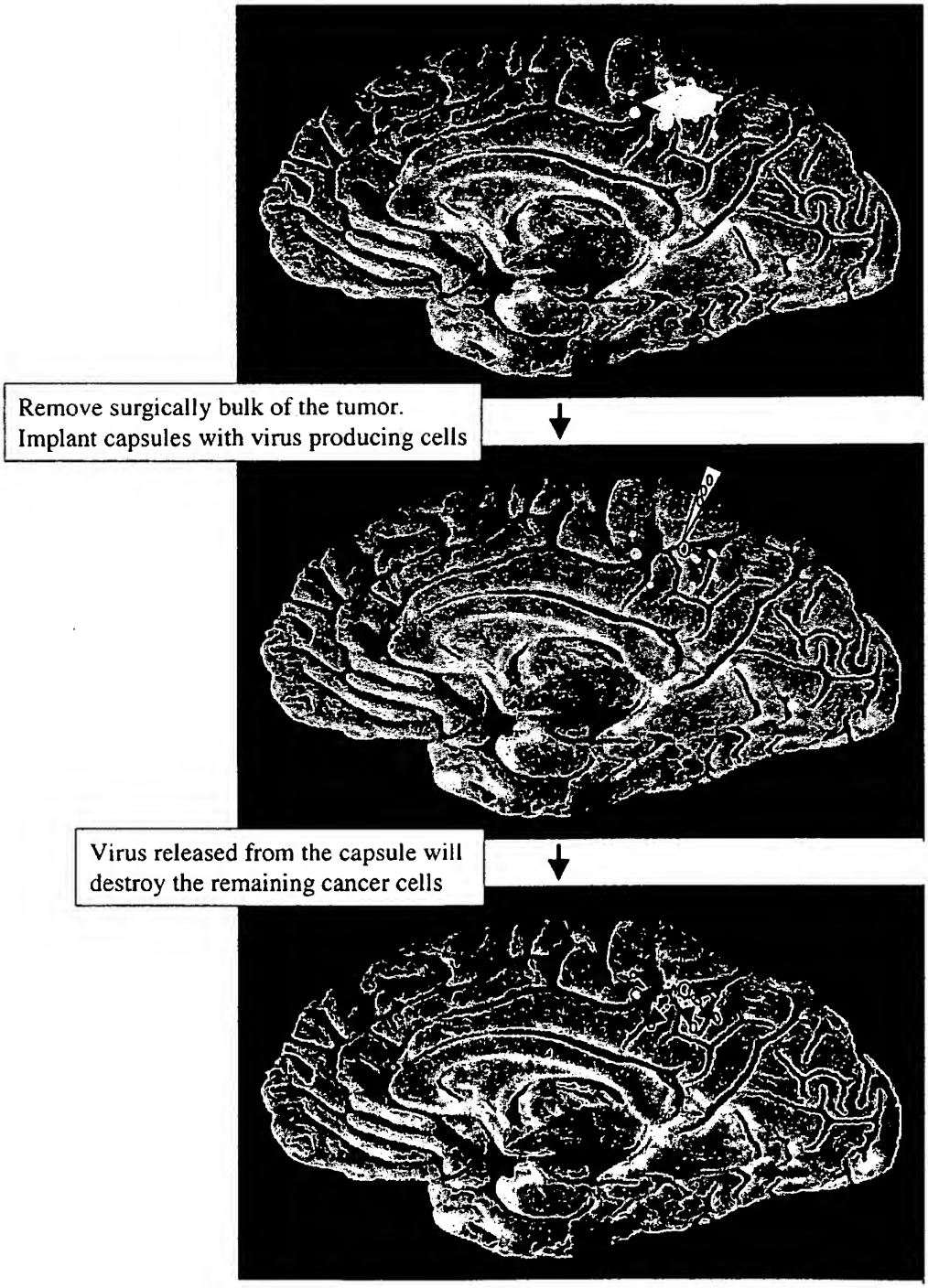

Fig. 2. Implantation of encapsulated virus-producing cells into brain.

systems with broad bystander effects, and/or systems that incorporate rapid killing of tumor cells, is extremely important in the treatment of large tumors.

In conclusion, gene therapy remains a promising tool for treating glioblastoma, especially in light of recent developments and findings. It is most likely that combinations of two or more gene therapy approaches (e.g., antiangiogenesis with oncolytic viruses) in conjunction with other methods such as surgery, radiation, and drug therapy will produce optimal results. Development of new strategies and delivery systems continues and the cure for one of the most deadly human diseases, glioblastoma, may yet be reached in the not too distant future.

REFERENCES

Alemany, R., Gomez-Manzano, C., Balague, C., Yung W. K., Curiel, D. T., Kyritsis, A. P., and Fueyo, J. (1999). Gene therapy for gliomas: Molecular targets, adenoviral vectors, and oncolytic adenoviruses. *Exp. Cell Res.* **252:**1–12.

Bansal, K., and Engelhard, H. H. (2000). Gene therapy for brain tumors. *Curr. Oncol. Rep.* **2:**463–472.

Bischoff, J. R., Kirn, D. H., and Williams, A. (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **274:**373–376.

Cheney, I. W., Neuteboom, S. T., Vaillancourt, M. T., Ramachandra, M., and Bookstein, R. (1999). Adenovirus-mediated gene transfer of MMAC1/PTEN to glioblastoma cells inhibits S phase entry by the recruitment of p27Kip1 into cyclin E/CDK2 complexes. *Cancer Res.* **59:**2318–2323.

Fueyo, J., Gomez-Manzano, C., Yung, W. K., and Kyritsis A. P. (1999). Targeting in gene therapy for gliomas. *Arch. Neurol.* **56:**445–448.

Gallay, P., Swingler, S., Song, J., Bushman, F., and Trono, D. (1995). HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83:**569–576.

Goldbrunner, R. H., Wagner, S., Roosen, K., and Tonn, J. C. (2000). Models for assessment of angiogenesis in gliomas. *J. Neurooncol.* **50:**53–62.

Gomez-Manzano, C., Fueyo, J., Alameda, F., Kyritsis, A. P., and Yung, W. K. (1999). Gene therapy for gliomas: p53 and E2F-1 proteins and the target of apoptosis. *Int. J. Mol. Med.* **3:**81–85.

Gupta, N. (2000). Current status of viral gene therapy for brain tumours. *Expert Opin. Investig. Drugs* **9:**713–726.

Haisma, H. J., Grill, J., Curiel, D. T., Hoogeland, S., van Beusechem, V. W., Pinedo, H. M., and Gerritsen, W. R. (2000). Targeting of adenoviral vectors through a bispecific single-chain antibody. *Cancer Gene Ther.* **7:**901–904.

Haroun, R. I., and Brem H. (2000). Local drug delivery. *Curr. Opin. Oncol.* **12:**187–193.

Hochberg, F. H., and Pruitt, A. (1980). Assumptions in the radiotherapy of glioblastoma. *Neurology* **30:**907–911.

Hughes, C., Galea-Lauri, J., Farzaneh, F., and Darling, D. (2001). Streptavidin paramagnetic particles provide a choice of three affinity-based capture and magnetic concentration strategies for retroviral vectors. *Mol. Ther.* **3:**623–630.

Jagus, R., Joshi, B., and Barber, G. N. (1999). PKR, apoptosis and cancer. *Int. J. Biochem. Cell. Biol.* **31:**123–138.

Ichikawa, T., Tamiya, T., Adachi, Y., Ono, Y., Matsumoto, K., Furuta, T., Yoshida, Y., Hamada, H., and Ohmoto, T. (2000). In vivo efficacy and toxicity of 5-fluorocytosine/cytosine deaminase gene therapy for malignant gliomas mediated by adenovirus. *Cancer Gene Ther.* **7:**74–82.

Karpati, G., Li, H., and Nalbantoglu, J. (1999). Molecular therapy for glioblastoma. *Curr. Opin. Mol. Ther.* **5:**545–552.

Maher, E. A., Furnari, F. B., Bachoo, R. M., Rowitch, D. H., Louis, D. N., Cavenee, W. K., and DePinho, R. A. (2001). Malignant glioma: Genetics and biology of a grave matter. *Genes Dev.* **15:**1311–1333.

Manche, L., Green, S. R., Schmedt, C., and Mathews, M. B. (1992). Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol. Cell. Biol.* **12:**5238–5248.

Mineta, T., Rabkin, S. D., Yazaki, T., Hunter, W. D., and Martuza, R. L. (1995). Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat. Med.* **9:**938–994.

Naldini, L., and Verma, I. M. (2000). Lentiviral vectors. *Adv. Virus Res.* **55:**599–609.

Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., and Huang, H. J. (1994). A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc. Natl. Acad. Sci. U.S.A.* **91:**7727–7731.

Pandori, M. W., and Sano, T. (2000). Photoactivatable retroviral vectors: A strategy for targeted gene delivery. *Gene Ther.* **7:**1999–2006.

Parney, I. F., Hao, C., and Petruk, K. C. (2000). Glioma immunology and immunotherapy. *Neurosurgery* **46:**778–792.

Puduvalli, V. K., and Sawaya, R. (2000). Antiangiogenesis—therapeutic strategies and clinical implications for brain tumors. *J. Neurooncol.* **50:**189–200.

Rainov, N. G. (2000). A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovirgene therapy as an adjuvant to surgical resection and radiation in adults with previous-lyuntreated glioblastoma multiforme. *Hum. Gene Ther.* **11:**2389–2401.

Read, T. A., Sorensen, D. R., Mahesparan, R., Enger, P. O., Timpl, R., Olsen, B. R., Hjelstuen, M. H., Haraldseth, O., and Bjerkvig, R. (2001). Local endostatin treatment of gliomas administered by microencapsulated producer cells. *Nat. Biotechnol.* **19:**29–34.

Sasaki, M., Wizigmann-Voos S., Risau, W., and Plate, K. H. (1999). Retrovirus producer cells encoding antisense VEGF prolong survival of rats with intracranial GS9L gliomas. *Int. J. Dev. Neurosci.* **17:**579–591.

Shir, A., Levitzki, A. (2001). Selective Inhibition of Glioma Growth by Tumor Specific Activation of Double-Stranded RNA dependent Protein Kinase-PKR. *Nat. Biotechnol.*

Wen, S., Stolarov, J., Myers, M. P., Su, J. D., Wigler, M. H., Tonks, N. K., and Durden, D. L. (2001). PTEN controls tumor-induced angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **98:**4622–4627.

Wick, W., Furnari, F. B., Naumann, U., Cavenee, W. K., and Weller, M. (1999). PTEN gene transfer in human malignant glioma: Sensitization to irradiation and CD95L-induced apoptosis. Oncogene **18:**3936–3943.

Wilcox, M. E., Yang, W., Senger, D., Rewcastle, N. B., Morris, D. G., Brasher, P. M., Shi, Z. Q., Johnston, R. N., Nishikawa, S., Lee, P. W., and Forsyth, P. A. (2001). Reovirus as an oncolytic agent against experimental human malignant gliomas. *J. Natl. Cancer Inst.* **93:**903–912.

Wu, S., and Kaufman, R. J. (1997). A model for the double-stranded RNA (dsRNA)-dependent dimerization and activation of the dsRNA-activated protein kinase PKR. *J. Biol. Chem.* **272:**1291–1296.

Zheng, S. X., Zhou, L. J., Zhu, X. Z., and Jin, Y. X. (2000). Antisense oligodeoxynucleotide inhibits vascular endothelial growth factor in human glioma cells. *Acta Pharmacol. Sin.* **21:**211–214.

Xu, K., Ma, H., McCown, T. J., Verma, I. M., and Kafri, T. (2001). Generation of a stable cell line producing high-titer self-inactivating lentiviral vectors. *Mol. Ther.* **3:**97–104.